THE NUTRITIONAL REQUIREMENTS

OF SPIRILLUM SERPENS

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THE NUTRITIONAL REQUIREMENTS OF *SPIRILLUM SERPENS*

DISSERTATION

Presented to the Faculty of the Graduate School of
The University of Texas in Partial Fulfillment
of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY

By

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PREFACE

The author wishes to express his deep appreciation to Professor Roger J. Williams for advice and encouragement during the course of this research. Appreciation is also due Professor I. M. Lewis, under whose direction preliminary studies were made. Funds for carrying out this work were provided by Standard Brands, Inc., of New York.

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Chapter I

INTRODUCTION

The fact that all of the known water-soluble "vitamins" required for the well-being of man and animals affect the growth of micro-organisms when tested under proper cultural conditions has made the field of bacterial nutrition of prime importance to the biochemist. Not only may the various organisms be used in assay methods for the known vitamins[1], but the study of the as-yet-unknown requirements of micro-organisms may provide us with a knowledge of new compounds necessary to the life of higher organisms. This has been amply demonstrated in the case of pantothenic acid[2], biotin,[2] and inositol[3].

[1] Studies on the Vitamin Content of Tissues. The University of Texas Publication No. 4137. Oct. 1, 1941.

[2] R. J. Williams, Biological Reviews, 16, 49 (1941).

[3] D. W. Woolley, Science, 92, 384 (1940).

During the past ten years Professor I. M. Lewis and his associates in the Department of Bacteriology of the Uni-

versity of Texas have isolated and cultured a number of species of bacteria of the genus Spirillum. Preliminary examination by members of that department indicated that of 28 strains investigated all but four could be grown on synthetic media composed of mineral salts and organic compounds. The investigation of the nutritional requirements of one of the remaining four organisms, identified as a strain of Spirillum serpens, was undertaken by the author.

Preliminary studies showed that while the organism could not be grown on simple synthetic media, the addition to the media of small amounts of natural extracts such as yeast extract or liver extract caused growth to take place. The effect of these extracts could not be duplicated by any of a large number of known bacterial nutrilites, including purine bases. Work was therefore undertaken on the isolation and identification of the substance or substances required for the growth of the organism. As the work neared completion the accumulated evidence as to the nature of the active material prompted a reinvestigation of the activity of purine and pyrimidine compounds. When this was made it was found that hypoxanthine, which previously had not been available, was physiologically active. Adenine and guanine, which had been tested before, were found to be active under certain conditions. All of the

activity of natural extracts could be accounted for by these compounds. The work leading to these conclusions is embodied in the following dissertation.

Chapter II

EXPERIMENTAL

A. Assay Method.

The assay method used may be considered a modification of the general microbiological assay technique developed in the laboratories of Professor R. J. Williams.[4] In general, this technique consists of culturing a micro-organism which requires for growth the particular active principle being investigated in a basal medium devoid of the active principle. As far as can be determined this medium is made complete in all other respects. No growth takes place in this basal medium alone, and the growth resulting from the addition to the medium of various extracts is a measure of the amount of active nutrilite present in those extracts, and can be compared with the growth produced by the addition of standard substances.

[4] The University of Texas Publication No. 4137. op. cit.

Organism.--The organism investigated was a strain of Spirillum isolated by the Bacteriology Department of the University of Texas and carried by them as Spirillum serpens F 4. Stock cultures were carried on nutrient agar slants (0.3 per cent meat extract, 0.5 per cent peptone,

1.5 per cent agar). These were inoculated from broth cultures, incubated for several days at 30°C., and stored in the refrigerator. Stock cultures were transferred every two weeks to fresh slants. Inoculum for daily assays was prepared by transferring from a stock culture to a sterile tube of meat extract-peptone broth (0.3 per cent meat extract, 0.5 per cent peptone). This inoculum was incubated at 30°C. for 20 hours before use.

Basal Media.--The basal medium used in preliminary experiments had the following composition:

	Amount in one liter of medium
Acid-hydrolyzed casein (vitamin-free)	5.0 g.
Inorganic salts	
K_2HPO_4	0.5 g.
KH_2PO_4	0.5 g.
$MgSO_4.7H_2O$	0.2 g.
NaCl	0.01 g.
$FeSO_4.7H_2O$	0.01 g.
$MnSO_4.4H_2O$	0.01 g.
Riboflavin	100 γ
Calcium Pantothenate	100 γ
Pyridoxin hydrochloride	100 γ

Thiamin chloride.	100 γ
Nicotinic acid.	100 γ
Biotin concentrate. . . .	Equivalent to 0.1 γ pure biotin

The pH was adjusted to 7.2-7.4.

When purified active material was available it was found that a much simpler medium than the above was satisfactory. The medium which was used in the majority of the experiments consisted of a solution containing 0.5 per cent l-asparagine and the inorganic salts listed above. No vitamin supplements were required. The asparagine in this medium could be replaced by l-aspartic acid or l-glutamic acid, but not by l-glutamine, glycine, d,l-alanine, tryptophane, urea, ammonium succinate, or sucrose plus ammonium sulfate.

The addition of 0.1 per cent of glucose or fructose to the medium caused complete inhibition of growth. Sucrose had no effect. Since the organism is strictly aerobic the inhibition caused by glucose and fructose is probably due to the reducing action of these sugars.

Standard Material.--The material used as a standard of activity before the identity of the active compounds was known was a sample of Difco yeast extract lot No. 332438. The growth-promoting activity of one mg. of this material was designated as one mg. unit.

Procedure.--Assays were carried out in 50 ml. Erlenmeyer flasks. These were conveniently carried in a metal tray that could be autoclaved. Solutions of the standard material or samples to be assayed were pipetted into the flasks in volumes up to 5.0 ml. The volume in each flask was then adjusted to 5.0 ml. by adding the appropriate amount of distilled water. To each flask was then added 22.0 ml. of the basal medium described above. (A total of 27.0 ml. was needed to fill the absorption cell used in measuring growth.) It was found not to be necessary to plug the flasks with cotton before sterilizing, but usually a clean cotton towel was placed over the flasks before sterilizing and left there during the incubation period. Sterilization was effected by heating for 10 minutes in the autoclave at 15 pounds steam pressure. After cooling to room temperature the flasks were ready for inoculation.

The cells from a 20-hour inoculum culture grown as described above were centrifuged out aseptically and resuspended in the original volume of sterile water. One drop of the resulting cell suspension was used to inoculate each flask. The flasks were incubated at 30°C. for 12 to 24 hours.

Measurement of Growth.--The growth response of the organism was measured by quantitatively comparing turbidities of the cultures in the thermoelectric turbidimeter described by Williams, McAlister and Roehm.[5] An absorption cell 50.0 mm. in length and having a volume of 27 ml. was used. The turbidity of the cultures was read on an arbitrary galvanometer scale. In practice this scale was so adjusted that zero represented the light (or heat) passing through a Corning AKLO No. 395 heat absorbing filter, and 100 represented no light (or heat) falling on the thermocouple. On such a scale the 50 mm. absorption cell filled with distilled water gave a reading of approximately 22. The following table illustrates the typical growth response produced by the standard yeast extract.

[5] Williams, McAlister and Roehm. J. Biol. Chem., 83, 315 (1929).

Table I

Mg. of yeast extract per 27 ml. culture	Galvanometer Reading
0	31.8
0.6	41.2
1.2	47.0
1.8	53.0
2.4	56.8
3.0	59.0

B. Effect of Various Known Compounds.

The following compounds were tested and found to be without activity: (uracil, adenine sulfate, guanine, xanthine, thymine), (acetyl choline), (inositol, pimelic acid, β-alanine, nicotinamide, thiamin pyrophosphate), (p-aminobenzoic acid), (ascorbic acid), (glutamine, asparagine, aspartic acid, histidine, creatine), (glyceraldehyde). The compounds were tested in groups as indicated by the parentheses and also in a mixture of all compounds together. The concentration of each compound was 10 μg. per ml. of media except in the case of nicotinamide and thiamin pyrophosphate, where the concentration was 0.1 μg. per ml.

As will be described later, adenine sulfate and guanine are active under certain conditions. Unfortunately, in the above experiment conditions were such as to entirely mask the activity.

Glucose, fructose, and sodium lactate completely inhibited the growth of the organism.

C. Source Materials.--In considering a source from which to concentrate the active substance or substances, assays were made on a number of available materials. The following table gives a list of such assays.

Table II. Source Materials

	Mg. units/mg.
Difco yeast extract	1.0 (standard)
Lilly's liver extract 373	0.25
Difco meat extract	2.2
Difco milk powder	inactive
Egg yolk	trace
Egg white	inactive
Urine	trace
Spinach extract	0.25
Wilson's liver fraction B	1.2-1.6
Parke-Davis rice bran extract	trace
Swift's dried meat scrap	0.45
Peptone	0.24
Animal tissues	
Rat liver$^{\Delta}$	0.22-0.36
Rat kidney$^{\Delta}$	0.15-0.20
Rat spleen$^{\Delta}$	0.17
Rat muscle$^{\Delta}$	0.15±
Rat heart$^{\Delta}$	0.07±
Rat brain$^{\Delta}$	inactive
Beef liver$^{\Delta}$	0.1
Beef muscle$^{\Delta}$	0.3

Bacterial tissues

Pseudomonas fluorescens[Δ]	inactive
Clostridium butylicum[Δ]	1.03

[Δ]These samples were hydrolyzed with one per cent. of their weight of the enzyme preparations clarase and caroid at a pH of 4.5 and a temperature of 37°C.

From the point of view of potency, availability, and cost Wilson's Liver Fraction B appeared to be the most desirable source material, and concentration procedures were carried out on this product.

D. Concentration Procedures.

The various procedures which were used in efforts to concentrate the activity of the liver extract are listed below, together with the results of such experiments.

(1) Adsorption on charcoal.... activity strongly adsorbed.

(2) Elution from charcoal..... activity eluted with 10 per cent ammonium hydroxide or boiling 10 per cent aniline.

(3) Adsorption on fullers earth..... activity adsorbed from acid solution. Practically no adsorption at neutrality.

(4) Elution from fullers earth..... activity eluted with 10 per cent ammonium hydroxide.

(5) Adsorption on Decalso..... activity not adsorbed.

(6) Precipitation with basic lead acetate..... activity partially precipitated.

(7) Precipitation with lead acetate in acid solution..... activity not precipitated. Considerable inert material precipitated.

(8) Precipitation with phosphotungstic acid..... activity precipitated.

(9) Precipitation with silver nitrate..... activity precipitated both in acid and in ammoniacal solution.

(10) Decomposition of silver precipitate..... activity recovered quantitatively by decomposing the precipitate with hydrochloric acid.

(11) Precipitation with copper sulfate and sodium bisulfite..... activity precipitated.

(12) Decomposition of copper precipitate..... activity quantitatively recovered by decomposing the precipitate with hydrogen sulfide.

(13) Extraction into butanol... activity partially extracted by prolonged treatment.

From the above procedures the following sequence of treatments was used to concentrate the activity: adsorption on charcoal, elution with ammonium hydroxide, precipitation of inert material with lead acetate in acid solution, precipitation with silver nitrate in ammoniacal solution, decomposition of the silver precipitate with hydrochloric acid, precipitation with copper sulfate and sodium bisulfite,

decomposition of this precipitate with hydrogen sulfide, fractional crystallization from water and alcohol. These steps are described in detail below.

Charcoal Adsorption.--The charcoal used was Darco G 60. Adsorptions were carried out at a pH of 7.0 and in a five per cent solution of the liver concentrate. The degree of adsorption of the activity was a function of the amount of charcoal used. For example, 20 per cent by weight of charcoal adsorbed 93 per cent of the activity, 10 per cent of charcoal adsorbed 85 per cent, while five per cent of charcoal adsorbed only 80 per cent of the activity. However, elution from the smaller amounts of charcoal produced products of greater activity per unit weight of solids.

Table III

Per cent of charcoal used	Per cent of original activity in first eluate	Mg. units per mg. of solids in first eluate
20	68	9
10	64	17
5	53	23

It was felt that the decrease in yield was more than offset by the increase in potency, and five per cent of charcoal was used in all subsequent adsorptions.

The actual large scale adsorption was carried out by the Wilson Laboratories of Chicago, who also supplied the liver concentrate used as a source material. One hundred pounds (dry weight) of Wilson's liver fraction B No. 256 was dissolved in water to make a five per cent solution. This solution was treated with five pounds of charcoal (Darco G 60). The charcoal was filtered out, washed with water, dried, and shipped to this laboratory.

This product proved to be not nearly so active as had been expected from the above laboratory results. The total yield of activity in the first eluate was only 10 per cent of the original activity and the potency of the eluate was only 12.5 mg. units per mg.

Elution was effected by shaking the charcoal from the adsorption with five times its weight of cold 14 per cent ammonium hydroxide solution for 30 minutes and filtering. Further treatment of the charcoal with boiling 10 per cent aniline solution yielded very little more active material.

Data from a typical concentration will be used to indicate the quantitative aspects of the procedures.

Five hundred grams of the charcoal from Wilson Laboratories (corresponding to 10 kg. of liver extract) was eluted with 2500 ml. of 14 per cent ammonium hydroxide solution and filtered.

Assay: 1,500,000 mg. units; Volume: 3500 ml.; Solids: 120 g.

Precipitation with Lead Acetate.--The filtrate from the ammonium hydroxide elution was evaporated on the steam bath until most of the ammonia was removed. The volume was reduced to approximately one-half. The pH of the solution was adjusted to 5.0 with acetic acid, and an excess of a concentrated solution of lead acetate was added. The precipitate that formed was filtered out and discarded.

Filtrate. Assay: 1,580,000+ mg. units; Volume: 2000 ml.; Solids: 85 g.

Precipitation with Ammoniacal Silver Nitrate.--The filtrate from the lead acetate precipitation was made weakly ammoniacal, and an excess of an ammoniacal solution of silver nitrate was added. A dirty white precipitate formed and was filtered out. The filtrate was discarded.

The precipitate was decomposed by boiling with an excess of dilute hydrochloric acid. The silver chloride was filtered out and the filtrate was neutralized with sodium hydroxide.

Assay: 1,400,000 mg. units; Volume: 1200 ml.

Precipitation with Copper Sulfate and Sodium Bisulfite.--This procedure has been widely used as a means of precipitating purine bases.[6] The neutral filtrate from the previous step

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[6] M. Krüger and C. Wulff. Z. physiol. Chem. 20, 176 (1895).

was treated with an excess of copper sulfate and sodium bisulfite. Approximately 50 grams of each was required for every 100 grams of solids in the filtrate. A greyish-pink precipitate formed, and the mixture was brought to boiling and filtered. After thorough washing with water the precipitate was suspended in a 3 N solution of sulfuric acid and decomposed by boiling with hydrogen sulfide. The mixture was filtered while hot, and the excess sulfuric acid was removed with barium hydroxide. The barium sulfate was filtered out, and the neutral solution containing the activity was evaporated to dryness.

Assay: 1,300,000 mg. units; Solids: 23.7 g.

Fractional Crystallization from Water and Alcohol.--The dry powder obtained above was refluxed with 10 times its weight of water. A small amount of insoluble material was filtered out, and the filtrate was cooled to 0°C. and allowed to crystallize. The precipitate was filtered out and dried. It contained a considerable part of the activity.

Assay: 100,000 mg. units; Solids: 810 mg; Potency: 123 mg. units per mg.

The mother liquor was evaporated to dryness and the residue was refluxed with one-half the original volume of water. Insoluble material was filtered from the hot solution, and the filtrate was cooled to 0°C. and allowed to

crystallize. The solids were filtered out and the filtrate was discarded. The solids were then refluxed with absolute alcohol (100 ml. per g.). Approximately one-half the material was insoluble in the hot alcohol and was filtered out. This substance was physiologically inactive. The active material was obtained by evaporating the alcohol solution to dryness. After two more recrystallizations from water a final product was obtained which had a potency of approximately 220 mg. units per mg.

Assay: 176,000 mg. units; Solids: 800 mg.; Potency: 220 mg. units per mg.

This material was approximately 60 per cent as active as synthetic hypoxanthine (370 mg. units per mg.). Nitrogen determinations by the micro Dumas method gave values of 46.4 per cent and 45.2 per cent nitrogen. These values, as well as the physiological activity, indicated that the product might be a mixture of hypoxanthine and adenine. The calculated nitrogen content of adenine is 51.8 per cent, of hypoxanthine 41.2 per cent. This conclusion was made more plausible by the fact that Bruhns[7] found hypoxanthine

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[7] Gustav Bruhns. Z. physiol. Chem. 14, 535 (1890).

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and adenine frequently to separate from a mixture of the two as an equimolecular complex. This complex had a solubility

less than that of either of the components alone. The calculated nitrogen content of such a complex is 46.52 per cent.

Separation of Hypoxanthine and Adenine.--The procedure used to separate these two compounds from the above mixture was that described by Bruhns.[8] One hundred and

[8] Gustav Bruhns, op. cit.

seventy-seven mg. of the mixture was dissolved in 50 ml. of water, and the solution was acidified slightly with nitric acid. Three hundred mg. of sodium picrate in 10 ml. of water was added. The voluminous precipitate that formed was filtered out and recrystallized once from a 25 per cent solution of acetic acid in water. The picrate was decomposed by suspending it in 10 ml. of a 3 N sulfuric acid solution and repeatedly extracting the mixture with ether. The picric acid was gradually removed into the ether, and a colorless solution remained. On neutralizing this solution with ammonium hydroxide and cooling, white crystals separated. These were recrystallized several times from water, filtered, washed with cold alcohol and dried. The yield was 17 mg. The nitrogen content by the micro Dumas method was 50.2 per cent (calculated for adenine, $C_5H_5N_5$, 51.8 per cent). The physiological activity of the material was identical with that of synthetic adenine.

The original filtrate from which the picrate was removed was made slightly alkaline with ammonium hydroxide, brought to boiling, and 500 mg. of silver nitrate in ammoniacal solution was added. A heavy yellow precipitate formed. This was filtered out while hot and was washed repeatedly with boiling water. The precipitate was suspended in 20 ml. of a 20 per cent nitric acid solution and was recrystallized several times from this medium. The recrystallized silver compound, which was now pure white, was suspended in 10 ml. of dilute nitric and one ml. of concentrated hydrochloric acid was added. The mixture was brought to boiling, and the heavy precipitate of silver chloride was filtered out. The filtrate was neutralized with ammonium hydroxide. On cooling the solution a fine crystalline deposit settled out, which was removed and recrystallized several times from water. The yield was 36 mg. The nitrogen content by the micro Dumas method was 41.8 per cent (calculated for hypoxanthine, $C_5H_4N_4O$, 41.2 per cent). The physiological activity of the product was identical with that of synthetic hypoxanthine.

E. Physical and Chemical Characteristics of Active Material.

There are two physical constants of physiologically active compounds that can usually be measured even in crude mixtures of the compounds. These are the isoelectric point

(in the case of ampholytes) and the diffusion coefficient. In the present case it was to a large extent the results of these measurements that prompted a reinvestigation of the purine compounds.

Isoelectric Point.--The isoelectric point was determined in the electrical transport apparatus designed by Williams and Truesdail[9]. The results of three experiments indicated

[9] R. J. Williams and J. H. Truesdail. J. Am. Chem. Soc. 53, 4171 (1931).

that the activity accumulated in cells having a pH of 7.4 to 7.9.

Diffusion Coefficient.--The diffusion measurements were made in an apparatus similar to that described by Mouquin and Cathcart[10]. Diffusion was carried out in a 0.3 N sodium

[10] H. Mouquin and W. H. Cathcart. J. Am. Chem. Soc. 57, 1791 (1935).

acetate buffer at a pH of 6.8. The temperature was 30.0°C. The diffusion coefficient was calculated according to the equation

$$D = \frac{\log(c_1+c_2) - \log(c_1-c_2)}{kt}$$

given by McBain and Liu[11], where D is the diffusion coef-

[11] J. McBain and T. H. Liu. J. Am. Chem. Soc., 53, 59 (1931).

ficient, k is the diffusion cell constant, t is the time of diffusion, and c_1 and c_2 are the concentrations in the two halves of the diffusion cell at the end of diffusion. Triplicate determinations were made, giving values for D of 0.865, 0.867, and 0.772 cm^2 per day. The last value appeared to be in error, and the average value for D was taken as 0.866 cm^2 per day. This value, determined at a pH of 6.8, is not the true minimum diffusion coefficient since the substance was found to have an isoelectric point between pH of 7.4 and 7.9. Any molecular weight approximated from the observed diffusion coefficient will be somewhat low.

From an appraisal of the literature on diffusion measurements (see particularly Mehl and Schmidt[12]) the

[12] J. W. Mehl and C. L. A. Schmidt, University of California Publications in Physiology. Vol. 8, No. 13, pp. 165-188.

author has observed that the majority of compounds will satisfy the equation

$$D\sqrt{M} = K$$

where D is the diffusion coefficient in cm^2 per day, M is the molecular weight and K is a constant of the order of eight to nine. The molecular weight of the active substance calculated on this basis is of the order of 85 to 130.

Chemical Characteristics.--Aside from the work done on the concentration procedures, a few observations were made on the effect of various treatments on the activity of extracts. Prolonged boiling or autoclaving had no effect on the activity. Boiling in 6 N sulfuric acid or 1 N sodium hydroxide did not affect the activity of preparations. Treatment with hydrogen peroxide completely destroyed the activity.

F. Physiological Activity of the Purines.

Adenine and Guanine.--Neither adenine nor guanine alone is physiologically active, but a mixture of approximately equal parts of the two shows activity. If either component of the mixture is appreciably in excess of the other, toxic effects are observed, and if the imbalance is extreme the physiological activity is completely masked. The quantitative relationship between the two compounds is illustrated in Table IV.

Table IV
Physiological Activity of Adenine and Guanine
(Growth Expressed in Galvanometer Readings)

γg. guanine per 27 ml. culture	γg. adenine sulfate per 27 ml. culture						
	0	5	10	20	50	100	250
0	27.0	32.5	31.1	30.0	29.5	29.0	28.7
5	25.9	55.0	56.1	55.0	34.8	30.8	29.4
10	25.8	46.5	58.0	60.5	55.0	34.8	30.0
20	26.0	32.9	56.0	63.0	60.0	53.0	31.0
50	25.8	30.0	36.8	56.7	63.5	60.0	44.1
100	26.0	30.1	31.8	45.0	57.5	54.2	44.3
250	26.0	28.9	30.1	37.9	55.0	53.5	51.0

Neither the purine xanthine nor the pyrimidines uracil, cytosine or thymine have any effect on the action of adenine or guanine.

Hypoxanthine itself is active, but if a small amount of hypoxanthine is added to cultures containing adenine growth greater than that produced by hypoxanthine alone is observed. Under certain conditions hypoxanthine thus appears to "activate" adenine as does guanine. This effect is illustrated in Table V. Hypoxanthine has no "activating" effect on guanine.

Adenosine and yeast adenylic acid are inactive when tested in combination with guanine.

Hypoxanthine.--This compound alone is able to replace entirely the growth promoting activity of natural extracts. The addition of approximately 0.5 μg. of hypoxanthine per ml. of medium enables maximum growth of the organism to occur. No toxic effects are observed in high concentrations (tested up to 10 μg. per ml.).

The effect of adenine on the activity of hypoxanthine presents a complicated picture. Depending upon the relative concentrations of the two compounds, adenine may cause increased growth of the organism, may be toxic, or may have no effect whatsoever. These effects are illustrated in Table V.

If the concentration of adenine is equal to or less than the concentration of hypoxanthine, growth greater than that produced by the hypoxanthine alone occurs. If the adenine concentration is greater than that of hypoxanthine toxic effects are observed, and if the ratio of adenine to hypoxanthine becomes large the physiological activity of the latter compound is entirely masked. It should also be noted that when the concentrations of both factors are large the toxic effect of adenine is apparent at lower adenine-hypoxanthine ratios than at lower concentrations.

Table V

Hypoxanthine and Adenine

μg. Hypoxanthine per 27 ml. culture	μg. adenine added per 27 ml. culture						
	0	2	5	10	50	100	200
0	28.0 +	--	--	--	--	--	--
2	33.5	38.8	35.1	29.8	--	--	--
5	41.0	45.8	47.8	39.1	28.0	28.0	--
10	50.5	52.2	54.8	52.5	29.5	28.0	--
15	55.8	--	--	--	--	--	--
100	67.0	--	--	67.0	66.0	51.0	33.8

Table VI

Hypoxanthine and Guanine

μg. Hypoxanthine per 27 ml. culture	μg. guanine added per 27 ml. culture		
	0	10	200
0	29.7 +	--	--
3	38.2	30.5	28.2
6	48.5	37.8	29.0
12	59.9	60.0	30.0
30	64.0	64.0	33.0
100	65.8	--	--
250	65.8	--	65.0

+ Growth expressed in galvanometer readings.

Guanine (Table VI) inhibits the growth stimulated by hypoxanthine provided the concentration of guanine is greater than that of hypoxanthine. If the amount of hypoxanthine present is in excess of the amount of guanine, the latter factor has no effect on growth.

Uric acid, xanthine, and uracil have no effect on the physiological action of hypoxanthine.

G. Enzymatic Destruction of the Activity

A comparison was made of the activity of rat liver extracts prepared by three different methods. Previous to this experiment all animal tissue extracts had been prepared by a process of enzymatic hydrolysis. Minced samples of the tissues were covered with a sodium acetate-acetic acid buffer solution of pH 4.5 and mixed with amounts of the commercial enzyme preparations Caroid and Clarase equal to one per cent of the weight of the tissue. The mixtures were preserved with a few drops of toluene and incubated at 37°C. for 24 hours. They were then heated to boiling and the solid residue filtered out.

In addition to the above method, an extract of rat liver was prepared by allowing the minced tissue to autolyze under the influence of enzymes naturally present in the tissue. Incubation again was at 37°C. for 24 hours.

The third method of preparing an extract of rat liver consisted of boiling the tissue immediately after removal from the animal. By this means all action of enzymes naturally present in the tissue was destroyed.

Assays on these extracts showed that natural autolysis destroyed the active material present in liver. The results are given in Table VII.

Table VII

Assays on Rat Liver Extracts

Method of Preparing Extract	mg. units per mg. of liver
Boiled immediately after removal from animal.	0.082
Autolyzed, unbuffered.	0.034
Autolyzed, pH 4.5.	0.114
Enzyme hydrolyzed.	0.22

From the above results, it would appear that the activity is destroyed by the action of enzymes present in the liver. It will be observed that at a pH of 4.5 this action is considerably diminished.

It is possible that the unbuffered autolysis above became somewhat acid during the course of the action, for in other experiments in which the autolyzing mixture was maintained at a pH of 7 by a phosphate buffer, the activity was completely destroyed.

The activity of hypoxanthine added to an autolyzing liver mixture was completely destroyed. The "adenine-activating" value of guanine was destroyed by similar treatment. Only approximately 25 per cent of activity of added adenine was destroyed by the autolyzing liver.

It should be noted that all of the activity of liver could not be extracted by boiling water without the aid of enzyme treatment.

Autolyzing rat kidney tissue destroyed activity in a manner similar to the autolyzing liver.

Chapter III

DISCUSSION

The physiological importance of the purine and pyrimidine bases has been recognized for many years. In combination with the sugars ribose and desoxyribose and with phosphoric acid they constitute the nucleic acids, and thus are common constituents of living cells. One of the purines, adenine, has recently been recognized as a constituent of several important coenzymes.

The essential nature of these compounds for the growth of micro-organisms has been reported a number of times. Richardson[13] showed that uracil was essential for

[13] G. M. Richardson. Biochem. J. 30, 2185 (1936).

the anaerobic growth of certain strains of *Staphylococcus aureus*, but not for the aerobic growth of the organism. Möller[14] showed that adenine was required for the growth

[14] E. F. Möller. Z. physiol. Chem. 260, 246(1939).

of *Streptobacterium plantarum*, while Pappenheimer and Hottle[15]

[15] A. M. Pappenheimer, Jr. and G. A. Hottle. Proc. Soc. Expt'l. Biol. Med. 44, 645 (1940).

found adenine to be necessary for the growth of a strain of Group A hemolytic streptococci. In the latter case the adenine could be replaced by hypoxanthine, guanine, xanthine, guanylic acid or adenylic acid. Furthermore, it was observed that purines were not required by the organism if the carbon dioxide tension was maintained at a sufficiently high level. Snell and Mitchell[16] have recently reported

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[16] E. E. Snell and H. K. Mitchell. Proc. Nat. Acad. Sci. 27, 1 (1941).

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the requirements of several lactic acid bacteria for purine and pyrimidine bases. Streptococcus lactis was found to require adenine and thymine for growth. Guanine was essential for the growth of Leuconcstoc mesenteroides, while uracil stimulated the growth of the latter organism and of Lactobacillus arabinosis.

To the above list of organisms requiring purines for growth must now be added the strain of Spirillum serpens used in this investigation. In a medium devoid of purines no growth whatsoever takes place. The purine requirements of the organism may be met in either of two ways. Hypoxanthine alone can provide the requirement. Maximum growth of the organism takes place if the medium contains approximately one-half microgram of hypoxanthine per milliliter of medium, and the presence of more hypoxanthine has no

effect on growth. In the absence of hypoxanthine a mixture of approximately equal parts of adenine and guanine provide the purine requirements of the micro-organism. In this case, both compounds are necessary, and the relative amounts of the two are important. If either compound is present in excess of the other toxic effects are observed. The maximum growth produced by the equimolecular mixture of adenine and guanine is the same as that produced by hypoxanthine.

The inhibitory action of certain purines on the physiological activity of others has been described in the experimental part of this dissertation. A similar relationship has not hitherto been reported in the case of the purine requirements of micro-organisms. However, several examples of a similar phenomenon with other nutrilites have been reported. McAlwain[17] showed that pyridine-3-sulfonic acid

[17] H. McAlwain. Nature 146, 653 (1940).

and its amide interfered with the growth of organisms requiring nicotinic acid. p-Aminobenzoic in small amounts has been reported to overcome the bactericidal effects of sulfanilamide and its derivatives.[18]

[18] M. Landy and J. Wyeno. Proc. Soc. Expt'l. Biol. Med. 46, 59 (1941).

Snell[19] has reported that the sulfonic acid analogue

[19] E. E. Snell. J. Biol. Chem. 141, 121 (1941).

of pantothenic acid inhibited the growth of organisms which require this vitamin. The addition of excess pantothenic acid reversed the inhibition.

The toxic effect of the pyridine-3-sulfonic acid, the sulfanilamide compounds, and the "thiopantothenic" acid is believed due to the fact that they are structurally very similar to the corresponding naturally occurring nutrilites. The inhibitory substances are thus able to fit into the biochemical patterns normally occupied by the essential nutrilites. They are not, however, able to carry out whatever vital functions are performed by these compounds, and the metabolic processes of the organism are blocked at this point.

As has been mentioned certain purines were found to inhibit the growth-promoting activity of others. The simplest case is that of hypoxanthine and guanine. Guanine in excess is able to block out hypoxanthine and inhibit the growth of the organism, and an excess of hypoxanthine is able to overcome this inhibition. The relationships between adenine and guanine and between adenine and hypoxanthine are more complicated, for in these cases purines which are inhibitory in excess are stimulatory in lower

concentrations. In seeking to rationalize these results the following hypothesis is suggested. It is offered only to indicate a possible explanation of the results obtained.

Suppose that the purines function in a physiologically essential complex that contains two molecules of purine bases per molecule. (Several coenzymes are known to be dinucleotides). Then, according to the results obtained, this complex can function only if (1) both purines are hypoxanthine, (2) one purine is hypoxanthine and the other adenine, or (3) one purine is adenine and the other guanine. The complex cannot function physiologically if (1) both purines are adenine, (2) both purines are guanine, or (3) one purine is hypoxanthine and the other guanine. The purines xanthine and uric acid do not compete for places in the complex. Such an hypothesis is in agreement with all of the observations, and is useful in indicating possible answers to a number of questions. For example, on this assumption it can be seen how it is possible for an excess of adenine or guanine to be toxic while an equimolecular mixture of the two promotes growth, and how it is possible for an excess of adenine to inhibit the action of hypoxanthine while a lower concentration adds to the growth.

The organism investigated differs in one respect from other organisms known to require purines. All of the

bacteria mentioned in the first of this discussion have rather complex nutritional requirements. All of them require one or more of the water soluble vitamins. On the other hand the requirements of Spirillum serpens are very simple. Aside from the purines only inorganic salts and asparagine were used in the medium. Specificity of the asparagine requirement has not been investigated thoroughly. This compound could be replaced by aspartic or glutamic acids but not by a number of other amino acids. It may be that the dicarboxylic amino acid (or amide) structure is essential for growth of the organism.

The destruction of activity produced by autolyzing slices of liver and kidney is probably due to the action of the enzyme xanthine oxidase.[20] This enzyme, a flavo-

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[20] D. E. Green. Mechanisms of Biological Oxidations. Cambridge Press. 1940, 95-102.

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protein, is one of the most thoroughly studied of all oxidative enzymes, and has been highly purified. Hypoxanthine, guanine and adenine are all oxidized by means of this enzyme, presumably to uric acid, but the relative rates of oxidation differ greatly. The relative rates of oxidation of hypoxanthine, guanine, and adenine are reported to be in the order 100:4:1.

Chapter IV

SUMMARY

Preliminary studies on the nutritional requirements of a strain of spirilla classified as Spirillum serpens F4 indicated that this organism could not be grown in media of known composition. The addition to synthetic media of small amounts of natural extracts such as yeast or liver extract enabled the organism to grow. Liver extract was found to be a suitable source of the active principle and concentration procedures were carried out on this extract. These procedures culminated in the isolation and identification of adenine and hypoxanthine as the substances required for the growth of the organism. These purines accounted entirely for the growth-promoting activity of natural extract.

The purine requirements of the organism were met by hypoxanthine alone or by an equimolecular mixture of adenine and guanine. Excess amounts of adenine or guanine inhibited the growth of the organism.

Aside from the purines the nutritional requirements of the organism were found to be simple. Only inorganic salts and asparagine, aspartic acid, or glutamic acid were required in the media. It is possible that the dicarboxylic

amino acid (or amide) structure is essential to the growth of the organism since these compounds could not be replaced by a number of other amino acids. None of the water soluble vitamins were required by the organism.

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